Microsomal lipoamide reductase provides vitamin K epoxide reductase with reducing equivalents

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This study was undertaken to search for the endogenous dithiol cofactor of the reductases of the vitamin K cycle. As a starting point, the redox-active lipophilic endogenous compounds lipoic acid and lipoamide were looked at. The study shows that microsomes contain NADH-dependent lipoamide reductase activity. Reduced lipoamide stimulates microsomal vitamin K epoxide reduction with kinetics comparable with those for the synthetic dithiol dithiothreitol (DTT). Reduced lipoic acid shows higher (4-fold) K_m values. No reductase activity with lipoic acid was found to be present in microsomes or cytosol. The reduced-

lipoamide-stimulated vitamin K epoxide reductase is as sensitive to warfarin and salicylate inhibition as is the DTT-stimulated one. Both vitamin K epoxide reductase and lipoamide reductase activity are recovered in the rough microsomes. NADH/lipoamide-stimulated vitamin K epoxide reduction is uncoupled by traces of Triton X-100, suggesting that microsomal lipoamide reductase and vitamin K epoxide reductase are associated. The results suggest that the vitamin K cycle obtains reducing equivalents from NADH through microsomal lipoamide reductase.

INTRODUCTION

The cyclic interconversions of vitamin K to its hydroquinone, to vitamin K epoxide and back to vitamin K which take place in the rough endoplasmic reticulum of hepatocytes is known as the vitamin K cycle. The cycle provides the energy for the post-translational carboxylation reaction that converts glutamyl residues in substrate proteins (e.g. clotting factors) into γ -carboxyglutamates (see refs. [1] and [2] for reviews).

The two reduction steps, i.e. vitamin K epoxide to vitamin K and vitamin K to vitamin K hydroquinone, are catalysed by the enzyme vitamin K epoxide reductase. *In vitro*, the enzyme needs a dithiol to supply reducing equivalents, suggesting the presence of redox-active thiol groups in its catalytic centre [3]. The 4-hydroxycoumarin anticoagulants such as warfarin are potent inhibitors of this reductase. These compounds bind tightly, essentially irreversibly, to the oxidized enzyme thereby hampering the dithiol-dependent re-activation of the enzyme. On enzyme activation the bound anticoagulant dissociates [4–6]. Therefore the dithiol reducing agent plays a central role in the intrinsic activity of oral anticoagulants [6].

The nature of the endogenous dithiol supplier is not yet unequivocally established; reduced thioredoxin [7,8] and reduced protein disulphide isomerase (PDI) [9] have been suggested as candidates. Both are water-soluble polypeptides (12000 and 57000 Da respectively) containing redox-active pairs of thiols and are involved in various oxidation-reduction reactions [10–12]. However, although abundantly present in cytosol and the lumen of the endoplasmic reticulum [13], detergent is needed to stimulate the *in vitro* microsomal vitamin K (epoxide) reductase activity with thioredoxin and/or PDI [9,14] (H. H. W. Thijssen, Y. P. G. Janssen and H. T. M. Vervoort, unpublished work) suggesting limited access to the reductase.

We investigated the potency of the physiological and highly lipophilic 7,8-dithioctic acid derivatives lipoic acid and lipoamide (LA), as suppliers of the reducing equivalents for microsomal vitamin K epoxide reductase. The study shows that microsomes contain NADH-dependent LA reductase activity

which provides vitamin K epoxide reductase with reducing equivalents.

EXPERIMENTAL

Materials

Chemicals were obtained from commercial suppliers: vitamin K (phyloquinone), NADH, NADPH and dithiothreitol (DTT) from Merck (Darmstadt, Germany); DL-lipoic acid, DL-lipoamide (LA), bovine LA dehydrogenase and Triton X-100 from Sigma Chemicals (St. Louis, MO, U.S.A.). Vitamin K epoxide was prepared as described [15]. The epoxide was dissolved at 2 mg/ml in 1% Triton X-100 in Tris buffer. Lipoamide dithiol (LAH2) was prepared as follows: LA in ethyl acetate (0.1 M) was thoroughly shaken in the dark at room temperature with NaBH_4 in water. The progress of the reaction was followed by assaying the thiol content with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid). After completion, the organic layer was washed with diluted HCl and water. The organic phase was evaporated and the product dissolved in dimethyl sulphoxide (DMSO) to obtain a 0.2 M solution of LAH2.

Methods

Rat liver microsomes were prepared by fractional centrifugation as described [6]. Microsomes were resuspended in Tris buffer (0.02 M Tris, 0.15 M KCl, 1 M NaCl, pH 7.4) to contain 20 mg/ml protein. Protein was estimated by the Lowry method with BSA as standard. The microsomal preparation was stored at -70°C .

Microsomes were fractionated into rough and smooth fractions by using discontinuous sucrose/CsCl gradient centrifugation as described by Eriksson et al. [16]: 10 ml of the 10000 g supernatant of rat liver homogenate in 0.25 M sucrose was layered on top of the gradient formed by 2 ml of 1.3 M sucrose and 1 ml of 0.6 M sucrose in 15 mM CsCl and subjected to centrifugation at 100000 g (at r_{av}) for 90 min using a SW40 rotor. Both fractions, rough and smooth, were resuspended in Tris buffer and pelleted

at 100 000 *g* for 1 h. The following characteristics were found for microsomes and their smooth and rough subfractions respectively: RNA ($\mu\text{g/ml}$ of protein, [17]): 82, 67, 155; cytochrome P-450 (nmol/mg of protein, [18]): 0.91, 0.08, 0.76.

Microsomal vitamin K epoxide reductase activity was assayed as described [6]. The reaction mixture contained (in order of addition) 0.4 mg of microsomal protein, vitamin K epoxide (85 μM , 2 μl of the 1% Triton X-100 stock solution), 10 μl of DMSO with or without LA and Tris buffer to make a final volume of 0.1 ml. After a preincubation of 5 min at 30 °C, the reaction was started with either DTT (5 μl of a DTT solution in Tris buffer) or NADH (5 μl of a NADH solution in Tris buffer) when LA served as the dithiol donor. The reaction was allowed to proceed for 5–10 min and the vitamin K formed was assayed by h.p.l.c. [6]. For the case when LAH2 served as the dithiol cofactor, the reaction was started by the addition of 10 μl of LAH2 in DMSO. To study the effect of Triton X-100 on the reductase reactions, a stock solution of vitamin K epoxide in propan-2-ol (2 mg/ml) was used as the substrate. The detergent was the last component to be added to the reaction mixture. LA reductase activity was assayed as follows: a reaction mixture containing, in a total volume of 0.1 ml, 0.4 mg of microsomal protein or 0.1 unit of LA dehydrogenase, 10 μl of LA in DMSO and Tris buffer was preincubated for 5 min at 30 °C. The reaction was started by the addition of NADH in 5 μl of Tris buffer. After an incubation period of 5–10 min, the reaction was stopped by the addition of 0.1 ml of propan-2-ol. The supernatant (20 μl) was mixed with 1 ml of 0.4% Ellman's reagent in phosphate buffer, pH 7.0. The absorption at 412 nm was recorded after 10 min. DTT was used to construct a calibration curve.

The kinetic constants, K_m and V_{max} , were determined by fitting the data to the Michaelis–Menten equation using the software package GPAD (GraphPad Software).

RESULTS

Pilot experiments showed that LA (5 mM) plus NADH (2 mM) stimulate microsomal vitamin K epoxide reduction to 20–30% of the DTT (5 mM)-stimulated activity. The addition of bovine heart LA dehydrogenase (0.05 unit) or cytosolic fraction (0.5 mg of protein) resulted in a stimulation comparable with that of DTT. Lipoic acid and/or NADPH were not able to replace LA and/or NADH respectively. DT diaphorase could not act as a reductase for LA or lipoic acid.

The LA/NADH-stimulated microsomal vitamin K epoxide reductase was characterized in more detail. The reaction was linear with the amount of microsomal protein (range tested 0.2–0.8 mg) and also linear with time of incubation (30 min). The results of enzyme kinetics experiments are presented in Table 1. Reciprocal plots of vitamin K epoxide reduction with respect to [LA] and [NADH] gave straight lines. The apparent K_m values for LA and NADH were about 1 mM. The apparent V_{max} for LA reduction by rat liver microsomes was about 40 nmol/min per mg of protein and the K_m values were 1.2 and 2.4 mM (Table 1). NADH was inhibitory at concentrations higher than 5 mM. The apparent V_{max} of bovine heart LA dehydrogenase when tested under comparable conditions was about 300 nmol/min per mg, and the apparent K_m for LA was about 2 mM.

LA/NADH-stimulated vitamin K epoxide reduction was as sensitive to warfarin inhibition as the DTT-stimulated reaction (Table 2).

Microsomes were subjected to subfractionation into smooth and rough microsomes; both enzyme systems, vitamin K epoxide reductase and LA reductase, were recovered mainly in the rough microsomes (Table 3).

Pilot experiments showed that LA/NADH-stimulated microsomal vitamin K epoxide reduction is sensitive to detergent (Triton X-100); 0.05% Triton X-100 appeared to reduce the LA/NADH-stimulated activity by 70–80% compared with that in standard conditions (0.02% Triton X-100). In contrast, the DTT-stimulated reaction was enhanced about 1.5-fold. To investigate further the effect of Triton X-100, the standard assay procedure was adapted by using vitamin K epoxide in propan-2-ol (see the Experimental section). Under these conditions, the DTT-stimulated vitamin K epoxide reductase activity was about 0.6 as high (results not shown). The detergent was found to inhibit the LA/NADH- as well as the DTT-stimulated reaction, the former system being much more sensitive: 0.01% Triton X-100 caused 40% inhibition of the reaction stimulated by LA/NADH but hardly affected the DTT-stimulated one (Figure 1).

Table 1 LA- and NADH-dependent kinetics of the microsomal vitamin K epoxide reductase and LA reductase

The data are means of two experiments carried out in duplicate. LA kinetics were studied with 5 mM NADH, and NADH kinetics were studied with 10 mM LA. For further conditions, see the Experimental section.

	K_m (mM)	V_{max} (nmol/min per mg of protein)
Vitamin K epoxide reductase		
LA	1.1	0.16
NADH	1.0	0.19
LA reductase		
LA	2.4	41
NADH	1.2	36

Table 2 Effect of S-warfarin on vitamin K epoxide reductase

The data are results of one experiment carried out in duplicate. The following conditions were used: DTT, 5 mM; LA, 10 mM; NADH, 5 mM. The percentage value given in parentheses is the percentage of the DTT-stimulated activity, which equals 320 pmol of vitamin K formed/min per mg of protein.

[S-warfarin] (μM)	Vitamin K epoxide reductase (%)	
	DTT	LA/NADH
0	100	100 (34%)
0.16	52	64
0.32	21	28
0.48	13	10

Table 3 Microsomal location of vitamin K epoxide reductase and LA reductase

Vitamin K epoxide reductase and LA reductase activities are expressed as percentages of their activities in total microsomes. The results are means of two separate estimates carried out in duplicate.

Sample	Vitamin K epoxide reductase		
	DTT	LA/NADH	LA reductase
Microsomes	100	100	100
Smooth microsomes	32.2	47.8	56.0
Rough microsomes	88.7	147.4	138.5

Figure 1 Effect of Triton X-100 on microsomal vitamin K epoxide reductase and LA reductase activity

The data are means of two separate experiments carried out in duplicate. Vitamin K epoxide reductase: ○, DTT-stimulated; ●, LA/NADH-stimulated; □, LAH2-stimulated; △, LA reductase. For reaction conditions, see the Experimental section.

Table 4 DTT- and LAH2-dependent kinetics of microsomal vitamin K epoxide reduction

The data are means $\pm$ S.D. of four separate experiments carried out in duplicate. Reaction mixtures contained either 0.02% (i.e. standard) or 0.05% Triton X-100. The vitamin K epoxide concentration was 85 μ M.

	K_m (mM)	V_{max} (pmol/min per mg of protein)
Triton X-100 (0.02%)		
DTT	0.27 ± 0.06	307 ± 53
LAH2	0.54 ± 0.03	316 ± 40
Triton X-100 (0.05%)		
DTT	0.55 ± 0.08	463 ± 55
LAH2	0.60 ± 0.06	343 ± 40

Table 5 Effect of salicylate on microsomal vitamin K epoxide reductase and LA reductase activity

The data are means of two separate experiments carried out in duplicate. The reactions with DTT (1 mM) and LAH2 (1 mM) were in the presence of 0.05% Triton X-100, and those with LA/NADH (10 and 5 mM respectively) in the presence of 0.02% Triton X-100. LA reductase was estimated using 10 mM LA, 5 mM NADH and 0.05% Triton X-100.

[Salicylate] (mM)	Vitamin K epoxide reductase (%)			LA reductase (%)
	DTT	LAH2	LA/NADH	NADH
0	100	100	100	100
0.2	78	81	70	108
0.5	56	65	59	93
1.0	47	49	41	88

An inhibition pattern comparable with the DTT-stimulated reaction was seen with LAH2 as the dithiol cofactor (Figure 1) and for LA/NADH with additional LA reductase activity (LA dehydrogenase or cytosolic fraction) (results not shown). The microsomal NADH-dependent LA reductase was stimulated (Figure 1).

The comparative enzyme kinetics of the DTT- and LAH2-stimulated microsomal vitamin K epoxide reductase are presented in Table 4. It appeared that the kinetics for DTT depended on the amount of detergent: at higher Triton X-100 concentration (0.05 compared with 0.02%), the apparent K_m as well as the apparent V_{max} were higher. The LAH2-dependent reaction was not affected by detergent. The apparent K_m value for LAH2 was comparable with that for DTT at 0.05% Triton X-100. The apparent V_{max} of the LAH2-stimulated reaction was comparable with that of the DTT-stimulated reaction at 0.02% detergent. LAH2 at concentrations above 3 mM appeared to be inhibitory to the reaction. The kinetics of reduced lipoic acid showed comparable V_{max} , but higher K_m (2.14 ± 0.30 mM, $n = 4$).

It has been suggested that the coumarin-like activity of salicylate involves interaction with the supply of physiological reducing equivalents rather than direct inhibition of vitamin K epoxide reductase [14,19]. The effect of salicylate in the various reactions was therefore studied. The results are summarized in Table 5. Salicylate inhibited equally DTT-, LAH2- and LA/NADH-stimulated microsomal vitamin K epoxide reduction (IC_{50} about 1 mM). In contrast, microsomal NADH-dependent LA reductase (Table 5) as well as LA dehydrogenase-catalysed LA reduction (results not shown) appeared to be insensitive to salicylate.

DISCUSSION

One of the main results of the present study is the observation that liver microsomes contain LA reductase activity capable of transferring reducing equivalents from NADH via LAH2 to the reductase(s) of the vitamin K cycle. To conclude that LAH2 is the endogenous dithiol cofactor of vitamin K (epoxide) reductase is as yet premature, but the study does provide evidence in its favour. First, the incubation experiments in the presence of Triton X-100 suggest that there is an organized relationship between NADH-dependent LA reduction and vitamin K epoxide reductase. As has been reported by others [14], detergents have deleterious effects on *in vitro* microsomal vitamin K epoxide reduction, suggesting a requirement for a lipidic (membranous) environment. The LA/NADH-stimulated reduction of the epoxide appears to be far more sensitive to detergent than the DTT-stimulated reaction. The higher sensitivity is neither due to inhibition of the microsomal LA reductase (in fact, LA reduction is enhanced) nor to a reduced accessibility of vitamin K epoxide reductase to LAH2. A likely explanation is that the two systems, LA reductase and vitamin K epoxide reductase, are in close association within the microsomes. Detergent, even when present in small amounts, affects the complex, thereby preventing optimal donation of LAH2. The fact that, like vitamin K epoxide reductase, LA reductase is also located in the rough microsomes (Table 3) supports the suggestion of systems being associated. The apparent discrepancy of the effect of Triton X-100 on DTT-stimulated vitamin K epoxide reductase needs some comment; the activation that is seen when Triton X-100 is added to the reaction mixture as the vehicle for the epoxide substrate is probably associated with substrate accessibility to the enzyme [20]. Why this effect is absent from the LAH2-stimulated reaction (Table 4) is not clear.

The specificity of the LA/NADH-stimulated system is a second argument in support of LAH2 as the dithiol donor. Although reduced lipoic acid can serve as a dithiol donor ([3]; this study), no lipoic acid reductase activity, NADH- or NADPH-dependent, is found. Moreover, the affinity of microsomal vitamin K epoxide reductase for reduced lipoic acid is lower.

The relatively high K_m values of the system for LA and NADH

can be put forward as a point against the argument. However, one should bear in mind that the enzyme kinetics experiments were performed in crude microsomal preparations using the rather water-insoluble substrates, LA and LAH₂. However, the K_m of LA for heart LA dehydrogenase has been reported to be about 1 mM [21] which is comparable with our findings. Furthermore, if LA reductase and vitamin K epoxide reductase are indeed coupled, optimal transfer of LAH₂ should be ensured. LA/NADH-stimulated microsomal vitamin K epoxide reduction has also been found in human liver [22] and bovine liver microsomes.

Recently, Soute et al. [9] postulated that vitamin K (epoxide) reduction is coupled to the formation of disulphide bridges in nascent secretory proteins. The coupling is suggested to be mediated via the redox-active peptides PDI and thioredoxin. The model seems attractive, for instance, it would provide an explanation for the apparent high vitamin K epoxide reductase levels in secretory organs such as pancreas and salivary gland [23]. However, the experimental evidence for the model must be seriously questioned. To support vitamin K epoxide reductase by the suggested pathway, the (bovine) microsomal reaction system has to be solubilized almost completely, indicating that neither thioredoxin nor PDI would have easy access to the membrane-embedded reductase [9]. Furthermore, all the elements have to be added to the system even though PDI has been shown to be abundantly present in the lumen of the endoplasmic reticulum [13,24] [it should be noted that LA is readily washed out during microsome preparation (H. H. W. Thijssen, Y. P. G. Janssen and L. T. M. Vervoort, unpublished work)]. In addition, if the vitamin K cycle has a role in protein synthesis as proposed, warfarin therapy would probably encounter serious side effects.

Neither warfarin nor salicylate had a direct effect on LA reductase, whereas their inhibition of vitamin K epoxide reductase was by a direct effect on the enzyme. Recently, it was argued that salicylate inhibition involved interruption of the transfer of cytosolic reducing equivalents to the microsomal vitamin K epoxide reductase [14]. This was concluded from the observation that, with fresh cytosolic fraction as donor of reducing equivalents, this enzyme is much more sensitive to salicylate. However, a more likely explanation for this observation can be found in the fact that the salicylate inhibition depends on the concentration of the dithiol reducing agent [19] (H. H. W. Thijssen, Y. P. G. Janssen and L. T. M. Vervoort, unpublished work). The cytosol-stimulated reductase activity was less than 10 % of the DTT-stimulated reaction [14], indicating low levels of the undefined cytosolic dithiol reducing agent (LAH₂?)

The well-known biochemical function of lipoic acid is to

transfer acyl groups from thiamin to CoA. Reoxidation of the reduced lipoic acid is mediated by the flavoprotein LA dehydrogenase. The reactions are involved in the oxidation of pyruvate and other α -oxo acids (e.g. α -oxoglutarate) by the pyruvate (α -oxoglutarate) dehydrogenase complex [25]. In this complex, lipoic acid is linked via an amide bond to a lysine residue of lipoate acetyltransferase suggesting no cellular free LA. On the other hand, at least 10 % of total rat hepatocyte LA dehydrogenase activity has been reported to be cytosolic and to differ from mitochondrial LA dehydrogenase [26], suggesting cellular functions for LA and LAH₂ other than the mitochondrial ones, such as the provision of the vitamin K cycle with reducing equivalents.

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